



USSN 10/017,717  
Attorney's Docket No.104732001500  
(formerly: 34639-20015.00)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of  
Guy M. MILLER, et al.

) Mail Stop:

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Application No.: 10/017,717

) Group Art Unit: 1614

Filed: December 14, 2001

) Examiner: P. Spivak

For: COMPOSITIONS AND METHODS

) Confirmation No. 5287

FOR THE PREVENTION AND

TRATEMENT OF TISSUE ISCHEMIA

**DECLARATION OF SEKHAR BODDUPALLI, Ph.D.**  
**UNDER 37 CFR 1.132**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Sekhar Boddupalli, declare and affirm as follows:

1. I am currently employed as Vice President of Discovery at Galileo Pharmaceuticals, Inc. ("Galileo"), and have been employed by Galileo since July 2000.
2. I received a Masters of Science degree from the University of Hyderabad, India in 1983 in Life Sciences, a doctorate (Ph.D. degree) from the Indian Institute of Science, Bangalore, India, in 1989 in Biochemistry, and served as a Research Fellow in Biochemistry at the University of Texas Southwestern Medical Center at Dallas from 1989 to 1991.
3. I supervised the metabolite studies reported below.

Tocopherol Metabolites effective in protecting cells and tissues against death and/or damage associated with hypoxia and/or ischemia.

Studies were conducted at Galileo Pharmaceuticals to assess the ability of certain tocopherol derivatives and metabolites to protect against cell death associated with tissue ischemia, in *in vitro*

cell models that are predictive of *in vivo* activity, as well as in certain *in vivo* models of tissue ischemia.

### 1. High Glutamate-Induced Oxidative Stress (HGOS) Assay

In the HGOS assay, a cell line derived from substantia nigral neuronal cells is subjected to high glutamate as a stressor. Glutamate is an excitatory neurotransmitter that is released by nerve cells in the brain, normally in small quantities to produce cell signals, as well as in response to prolonged anoxia or ischemia. In this latter condition, the resulting high concentrations of glutamate have been shown to produce ischemic cell damage. The HGOS assay is set up to measure the ability of test compounds to prevent or reduce glutamate-induced cell damage in these cells. This assay is therefore recognized as a standard model of tissue damage.

Briefly, a dopaminergic neuronal cell line (SN-4742) derived from the substantia nigra of the brain was obtained from Cornell University Research Foundation and was grown in DMEM-No Glucose (Life Technologies, Inc., Rockville, MD; Cat # 11966-025) supplemented with 1.4 mM L-glutamine (Life Technologies), 29.1 mM D-glucose (Sigma ; St. Louis, MO), 100 U/ml penicillin and 1000 $\mu$ g/ml streptomycin (1xP/S ; Gibco/Invitrogen, Carlsbad, CA). Cells were seeded into 96-well plates (precoated with poly-D-Lysine; Corning) at a density of 2000 per well and left to grow for 72 hours in a 33°C incubator with 5% CO<sub>2</sub> in air atmosphere. The passage number of the cells for each assay experiment were no later than p11 in order to minimize experimental variation.

All compounds were dissolved in DMEM-No Glucose containing 1mM glucose, 30 mM glutamate and 1x Pen/Strep. DMEM-No Glucose with 1mM glucose and 1x P/S was used as the negative control, DMEM-No Glucose with 1mM glucose, 100 M glutamate (L-glutamic acid, monosodium salt; Sigma) was used as a positive control and 100 $\mu$ M Glutathione was added to the positive control as a standard. Compound solutions were loaded into VWRBrand DyNA Block 1000, deep well mother plates (VWR, West Chester, PA).

Incubation medium was removed from each well of the cell culture plates using standard aseptic techniques and an 8-channel aspirator, and cells were washed with 200 $\mu$ l of HBSS (0.244g/L MgCl<sub>2</sub>.6H<sub>2</sub>O, 0.373g/L KCl, 5.958g/L HEPES buffer, 5.844g/L NaCl, 0.136g/L KH<sub>2</sub>PO<sub>4</sub>, 0.191g/L CaCl<sub>2</sub> .2H<sub>2</sub>O, pH 7.4). The plates were then placed in the humidified 37°C incubators of a Biomek 2000 Side Loader Workstation (Beckman Instruments, Fullerton, CA).

Beckman Biomek workstations were used to remove the HBSS washing solution and to load the compounds and controls from the mother plates onto the cell plates. The plates were incubated with test compounds or controls at 37°C in 5% CO<sub>2</sub> for exactly 16 hrs. Compounds were removed from the plates, washed once with 200µM of pre-warmed 1x HBSS and then 100µL of 5µM Cell Tracker Green fluorescent dye (Molecular Probes, Eugene, OR) was added to each well. The plates were incubated at 37°C for 30 minutes. After washing the cells twice with prewarmed 1x HBSS, the plates were read with the 485 excitation; 538 emission filter pair using an automated fluorescent microscope/imaging system (Fluroscan; Universal Imaging, Downingtown PA). This dye labels live cells; thus, viability was measured relative to controls, by increased fluorescence levels.

Compounds are considered to be active against ischemic damage when they exhibit protection against HGOS cell injury and cell death in the assay described above. In studies carried out in support of the present invention, the following EC<sub>50</sub> values were determined: Gamma-tocopherol metabolite 2,7,8-trimethyl-2-(beta-carboxy-ethyl)-6-hydroxy chroman (LLU-alpha; gamma-CEHC), 49.5 micromolar; delta-tocopherol metabolite 2,8-dimethyl-2-(beta-carboxy-ethyl)-6-hydroxy chroman [GPI-20694; delta-CEHC], 49.3 micromolar; gamma-tocopherol metabolite 2,3-Dimethyl-5-[2-(2-methyl-5-oxo-tetrahydro-furan-2-yl)-ethyl]-[1,4]benzoquinone, 3.0 micromolar.

These data support our invention by showing that metabolites of non-alpha tocopherols are protective against ischemic cell death.

## 2. MPP<sup>+</sup> Cell Death Assay

The MPP<sup>+</sup> cell death assay is an alternative assay for assessing protection against ischemic cell death. This assay measures the ability of test compounds to prevent cell death due to insult with the neurotoxin 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>). This compound is the oxidized form of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), which, if administered to humans or animals, destroys cells into which it is selectively taken up. MPP<sup>+</sup> concentrates in the mitochondria of cells equipped with dopamine uptake mechanisms and is thought to act via interference with mitochondrial respiration in the respiratory chain, similar to other respiratory poisons such as rotenone. Accordingly, compounds that are protective against MPP<sup>+</sup> toxicity are deemed to protective against cell death associated with ischemia or hypoxia.

Cells were seeded in poly-D-lysine-coated 24-well plates at a density of 4500 cells per well in the 10% serum medium described below. Briefly, neuronal cells, as described above, were grown in DMEM-No glucose, supplemented with glucose (29.1mM), L-glutamine (1.4mM), 10% heat-inactivated fetal bovine serum (FBS), and 1x penicillin/streptomycin (P/S), and were washed with DMEM-No glucose and 1x P/S. The cells were left to attach for 16 hours in a 33°C incubator (5% CO<sub>2</sub>), after which they were washed once with 500µL wash media and then differentiated into a neuronal phenotype by incubating in Low Serum Medium (DMEM-No glucose, supplemented with 1.4 mM L-glutamine, 0.5% FBS, and 1x P/S) for 24 hours in a 39°C incubator (5% CO<sub>2</sub>).

After 24 hours the low serum medium was aspirated from the cells and the monolayer was washed once with 500µL wash media. Test compounds were diluted to 2-fold the desired testing concentration in assay media and 250µL was added to the cells. From a 10mM stock, a working solution of 140µM 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) (Sigma, St. Louis, MO) was made in assay media and 250µL of this working solution was also added to the cells. The final volume in each well was 500µL and the final concentration of MPP<sup>+</sup> was 70µM. As a negative control, cells were incubated with 500µL assay media with no additions.

Cells were incubated in a 39°C incubator (5% CO<sub>2</sub>) for 24 hours. After this time, the number of live neurons remaining in each well was determined using a fluorescent vital cell stain, Cell Tracker Green (Molecular Probes, Eugene, OR). Assay media was aspirated from the cells and 400µL of 2.5 µM Cell Tracker Green was added to each well. Cells were placed in a 37°C incubator for 5 minutes after which time the cell stain was aspirated off and 500µL of HBSS (Invitrogen Life Technologies, Carlsbad, CA) was added to each well. The number of live cells in each well was then quantitated using an automated fluorescent microscope/imaging system (Fluroskan; Universal Imaging, Downingtown PA).

In this assay, the gamma-tocopherol metabolite 2,7,8-trimethyl-2-(beta-carboxy-ethyl)-6-hydroxy chroman (LLU-alpha) protected cells in this assay with an EC50 of 50.4 micromolar; the delta chroman metabolite 2,8-dimethyl-2-(beta-carboxy-ethyl)-6-hydroxy chroman protected cells with an EC50 of 27.9 micromolar.

These data further support our invention by showing that metabolites of non-alpha tocopherols are protective against ischemic or hypoxic cell death.

The foregoing assays are predictive of *in vivo* anti-ischemic activity, as exemplified in the specification. By way of example, compounds that were active in the cell models described above, such as, for example, gamma-CEHC, were found to be active in reducing ischemic cell damage and associated edema in brain tissue in the rat MCAO model described in Example 2.

I declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

Date: 1/14/04

By:   
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**DECLARATION OF GUY MILLER, M.D., Ph.D.**  
**UNDER 37 CFR 1.132**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Guy Miller, declare and affirm as follows:

1. I am currently employed as Chairman and Chief Executive Officer at Galileo Pharmaceuticals, Inc. ("Galileo"), and have been employed by Galileo since 1995.
2. I am an inventor and/or co-inventor of one or more of the pending claims in the above-referenced U.S. Patent application ("USSN 10/017,717").
3. I am also a co-inventor, with Lesley A. Brown, of the claims in U.S. Patent 6,528,042 B1, which issued on March 4, 2003.

I understand that U.S. Patent 6,528,042 has been cited by the Examiner in USSN 10/017,717 as a reference under 35 U.S.C. 102(e) on the grounds that it discloses certain information that is now claimed in USSN 10/017,717, which lists me, along with Lesley A. Brown and four other individuals as co-inventors.

Any information that may have been disclosed but not claimed in U.S. Patent 6,528,042 is attributable to Lesley A. Brown and me.

I declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

Date: 1-12-04

By:   
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